ACTION OF AQUEOUS EXTRACT OF BEEF SPLEEN ON CELLS OF SARCOMA 37 ASCITES[1]

IRENE COREY DILLER, GEORGE F. WATSON[2] AND MARY E. FISHER

Institute for Cancer Research, Philadelphia 11, Pa. and Marine Biological Laboratory, Woods Hole, Mass.

An inhibitory action of beef spleen extract on growth of sarcoma 180 and mammary carcinoma of the mouse has been reported by Lewisohn (1938, 1945); of mouse sarcoma 37 and methylcholanthrene-induced sarcomas by Diller and Watson (1949); and of Brown-Pearce carcinoma of the rabbit by Nuttini *et al.* (1951). The mouse tumors studied by Diller and Watson (1949) showed degenerative cell changes resulting in shrunken bean-shaped nuclei and vacuolated cytoplasm at certain concentrations and dose levels, but no damage could be detected in normal tissues of the treated mice. MacFarlane and collaborators (1948) reported similar degenerative changes when cells of certain spontaneous tumors were stored in concentrated aqueous spleen extract for varying lengths of time at 5° C., which led them to believe that spleen extract could have a direct cytolytic action. More recently Katzberg (1952) incubated fragments of sarcoma 180 and of normal mouse tissues at 37° C. in Parker's medium and in medium containing either Rockland mouse spleen extract or Rockland mouse spleen pulp. Both malignant and non-malignant tissues remained viable in Parker's medium for ten days or longer. Splenic preparations apparently produced an agent which accelerated cytolysis. Maximum sensitivity to this agent was shown by sarcoma 180 (total destruction of cells in 48 hours) and maximum resistance by normal tissues of the Rockland mouse.

Our experiments (1949) with trocar-implanted or chemically induced tumors indicated that regression was accompanied by a marked inflammatory reaction in the vicinity of the tumor and an upsurge of mitotic activity in the organs of the hemopoietic system; a direct destructive action could not, therefore, be ascribed to the splenic extracts which we employed. Neither do such *in vitro* experiments as those of Katzberg (1952) offer clear-cut proof of direct cytolytic action, since under the conditions of his experiments, untreated tumor tissues, as well as normal tissue controls, were undergoing degeneration from the outset, and the effect of the extract, though more rapid in the case of tumor tissue, was largely an acceleration of degenerative change. The most convincing evidence of direct effect in Katzberg's experiments was the occurrence of metaphase block in sarcoma 180 during the first 24 hours after exposure to his splenic preparations.

In the hope that additional light could be thrown on the action of spleen extract in direct contact with tumor cells, further experiments were carried out in this laboratory using the ascites form of sarcoma 37, the solid form of which had been studied previously in this connection (Diller and Watson, 1949).

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[2] Kitchener Clinic, Kitchener, Ontario, Canada.

Material and Methods

Albino mice, 6 to 8 weeks of age, both males and females, of the special Swiss strain maintained in the main colony of our Institute were inoculated intraperitoneally with one cc. of sarcoma 37 ascites tumor. The growth was allowed to develop for three days, when abdominal swelling could be observed visually. A sample of the ascites fluid (see Fig. 1) was withdrawn, diluted 1 : 200 in saline solution and counted in a hemacytometer. The animals were then injected intraperitoneally with a single dose (one cc.) or with 4 doses (0.25 cc. each) of varying concentrations (50 mg., 100 mg., and 150 mg. of solids/ml.) of crude aqueous cell-free, beef spleen extracts (not commercially available) obtained through the courtesy of the Eli Lilly Company, or prepared at the Kitchener Clinic and processed in Philadelphia by the McNeil Laboratories.[3] In the Kitchener Clinic the extract is prepared by mincing 400 grams of fresh calves' spleen under sterile conditions and extracting it in 1000 cc. of sterile saline at 33°–35° C. Approximately 400 cc. of the supernatant is decanted after four weeks and passed through a Seitz filter. Details of preparation and clinical use will be treated in a forthcoming paper under the authorship of the medical member of the group (G. F. W.). Control animals received corresponding amounts of sterile salt solution. The extracts were found to be very unstable, even though prepared and maintained constantly at temperatures just above freezing. Color change from red to dark brown usually accompanied loss of potency.

Ascitic fluid was withdrawn from each mouse at intervals of 6, 24 and 48 hours after spleen extract injection for hemacytometer counts. Similar counts were made of control mice that had received neither spleen extract nor saline solution, in order to determine whether the cell population was affected by repeated withdrawals. This was apparently not the case, since the cell count remained essentially static. Permanent preparations of each sample were fixed in Allen's or Carnoy's fluid and stained with Feulgen-fast green for differential counts. Detailed phase contrast studies were made also of living cells stained with neutral red to aid in detection of degenerating nuclei, after the method of Goldie and Felix (1951).

Observations

Cell count. The most striking result was the decrease in tumor cell population following spleen extract injection (see Table 1). This was accompanied by subsidence of the ascites swelling. Stained smears of the residual fluid (usually less than 0.5 cc. after 48 hours) revealed small clumps or islands of cells as indicated in Figure 3, which presumably served as centers for formation of solid masses. Some control animals also developed solid tumors or sheets of tumor cells on the peritoneum, but there was never any regression of the ascites swelling in untreated mice.

The reduction in number of tumor cells observed in our experiments was apparently in part the consequence of infiltration of leucocytes and histiocytes, by which the tumor cells were phagocytized, in part of cessation of mitosis, and in part of cellular degeneration (see table of differential counts).

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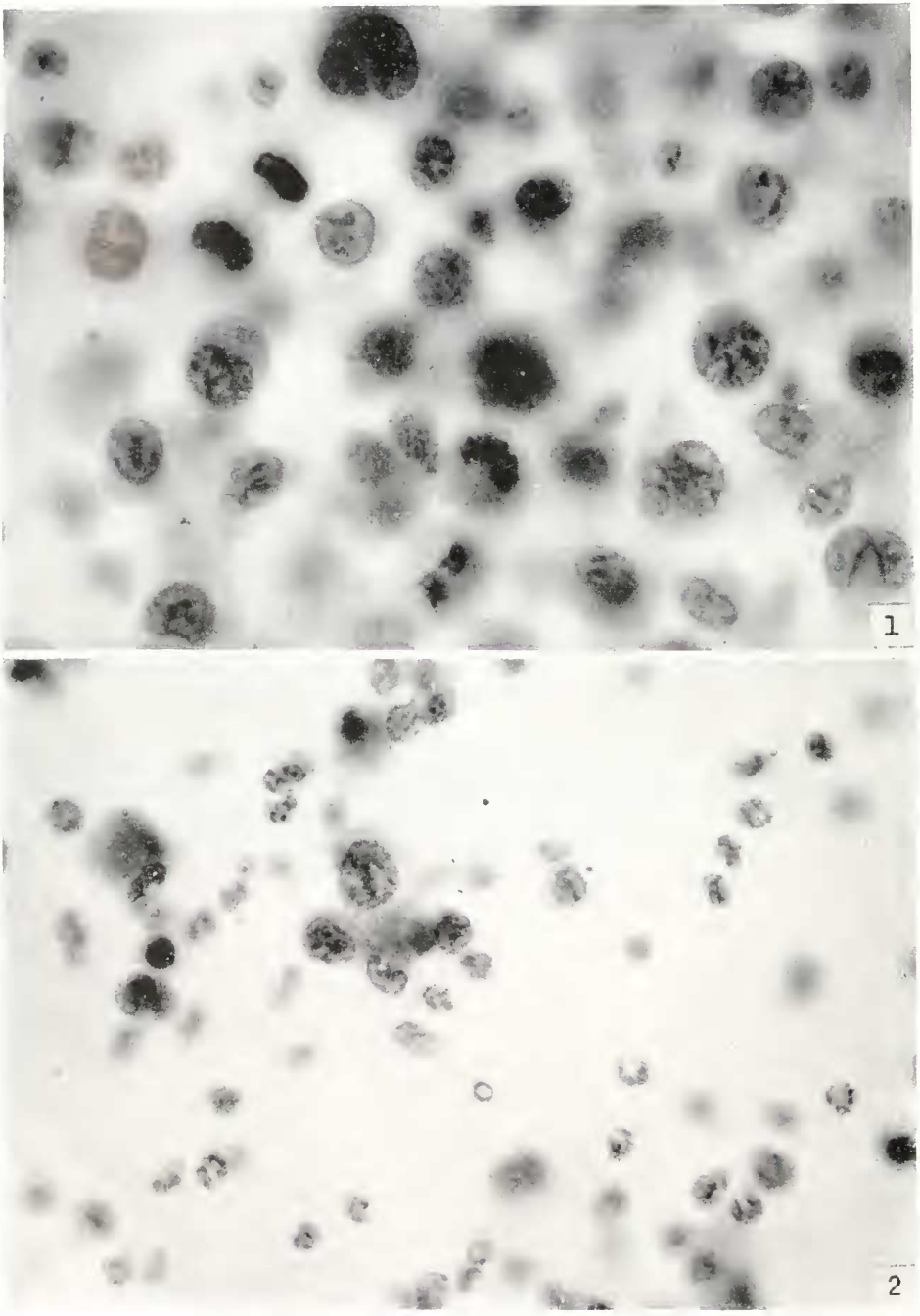


FIGS. 1–2.

Sarcoma 37 ascites tumor preparations from mice injected with aqueous beef spleen extract. Picric-formol acetic fixation, Feulgen-fast green.

TABLE I

Cell count per cc. of sarcoma 37 ascites fluid (Average of 20 animals)

	Spleen inj. One cc. of 150 mg. conc.	Saline inj. One cc.	Uninjected
Pre-inj.	50.2×10^6	54.0×10^6	54.9×10^6
6 hrs. post-inject.	30.2×10^6	53.5×10^6	54.5×10^6
24 hrs. post-inject.	21.2×10^6	50.3×10^6	53.7×10^6
48 hrs. post-inject.	26.0×10^6	51.7×10^6	51.2×10^6

Mitotic phenomena. Counts of 1000 cells per specimen were made on stained preparations of fluid withdrawn prior to spleen extract injection, and at 6 hours, 24 hours, 48 hours and 72 hours post-injection. An average of 3.6% of the tumor cells were in mitosis just before treatment. In many individuals no mitotic figures at all could be observed 6–24 hours after injection of spleen extract, and in all individuals there was a decreased percentage of cells in mitosis (see Table II). When one cc. of spleen extract (150 mg. of solid/cc.) was administered in a single dose, mitotic activity was suppressed for at least 48 hours, but if smaller doses of extract were given, it was necessary to repeat them at 6-hour intervals in order to achieve this result.

TABLE II

Differential count, based on 1000 cells per mouse per sample*

	Mitosis %				Necrosis %			Polymorphs %				Lymphs %				Tumor cells (non-dividing) %			
Treatment:	Pre	6 h	24 h	48 h	Pre	6 h	48 h	Pre	6 h	24 h	48 h	Pre	6 h	24 h	48 h	Pre	6 h	24 h	48 h
Spleen extract 50 mg./ml.	3.1	0.3	0.0	0.2	0.4	1.5	3.8	17.5	39.8	59.7	32.4	3.0	10.0	13.6	24.0	76.0	48.4	26.7	39.6
Spleen extract 150 mg./ml.	3.7	1.4	1.4	0.1	0.4	0.4	60.1	20.9	28.8	40.0	24.9	4.4	4.3	6.0	9.4	70.6	65.1	52.6	5.5
Saline inj.	3.9	3.5	3.6	3.7	1.1	0.2	1.5	20.3	16.8	18.1	19.4	8.5	7.5	9.7	6.1	66.2	72.0	68.6	69.3
Non-inj. controls	3.6	3.4	3.8	3.8	1.3	1.2	2.9	13.0	13.9	18.9	16.2	9.5	10.2	9.9	10.8	72.6	71.3	68.3	66.3

* 20 mice in each group.

Blood cell counts.

a. *In peritoneal fluid.* The number of polymorphonuclear leucocytes increased immediately after spleen injection in all cases (Fig. 2), and continued to rise for about 30 hours, then gradually returned to the level of the controls as shown in Table II. There was an accompanying increase in percentage of lymphocytes, which persisted into the 48-hour period. The lymphocytic count agrees with that

FIGURE 1. Smear preparation of untreated control, three days after inoculation into the peritoneal cavity. Ca. 800 ×.

FIGURE 2. Smear preparation of ascitic fluid withdrawn from the same mouse 24 hours after intraperitoneal injection of spleen extract (50 mg./ml.). Only two tumor cells remain in a comparable field; the remainder of the cell population is made up of leucocytes. Ca. 800 ×.

observed when solid tumors were treated with spleen extract as described in an earlier paper (Diller and Watson, 1949). Macrophages and other histiocytes were present also in the ascitic fluid, but there was no significant change in number of these cells following spleen injection, as was the case with solid tumors (Diller and Watson, 1949; Lewisohn, 1938, 1945).

b. *Peripheral blood.* A comparison of the effect of injection of spleen extract on the peripheral blood picture (tail vein samples) of tumor-bearing and non-tumor-bearing mice appears in Table III.

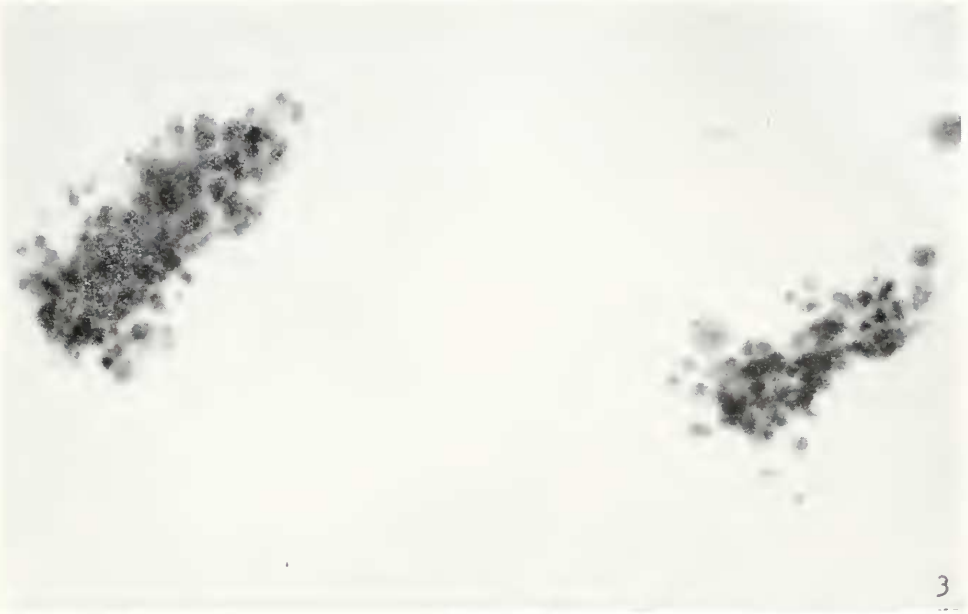
TABLE III

Peripheral (tail vein) leucocyte count (Average of 20 mice)

Mouse strain	Tumor- or non-tumor-bearing	Treatment	Route of inj.	Duration of treatment	W.B.C. count mm.^{3} 1:20 dil.
Swiss	Non-tumor	—	—	—	15,459
Swiss	Non-tumor	Spleen ext. 150 mg./cc. 1 cc. daily	I.P.	6 days	15,740
Swiss	S-37 solid 7 days post-implant.	—	—	—	21,990*
Swiss	S-37 solid 7 days post-implant	Spleen ext. 100 mg./cc. 1 cc. daily beg. 4th day post-implant.	I.P.	24 hrs. 72 hrs.	30,060 32,530
Swiss	S-37 ascites 5 days post-implant.	—	—	—	18,500
	S-37 ascites 5 days post-implant.	Spleen ext. 150 mg./cc. 1 cc. on 3rd day post-implant.	I.P.	48 hrs.	39,120

* Data from an earlier experiment (1949). Fekete in *Biology of the Laboratory Mouse*, 1941, p. 94, reported an average peripheral count (based on unpublished data of Law and Heston) of 21,510 for tumor-bearing Bagg albinos.

Percentage of viable tumor cells. The percentage of non-dividing tumor cells before and after injection, based on a count of 1000 cells per preparation, is also shown in Table II. Animals injected with the Lilly extract showed an average decrease in number of tumor cells from 76% before injection to 26.7% at 24 hours. This was apparently due to several factors—dispersion of cells by reason of infiltration of leucocytes, cessation of mitosis, and, as indicated by uninjected controls, to slight lowering of cell numbers by daily withdrawal of fluid. With the fall of the leucocyte count at 48 hours and resumption of mitosis, the tumor cell count per 1000 cells was increased to 39.6%. Following the injection of an extract



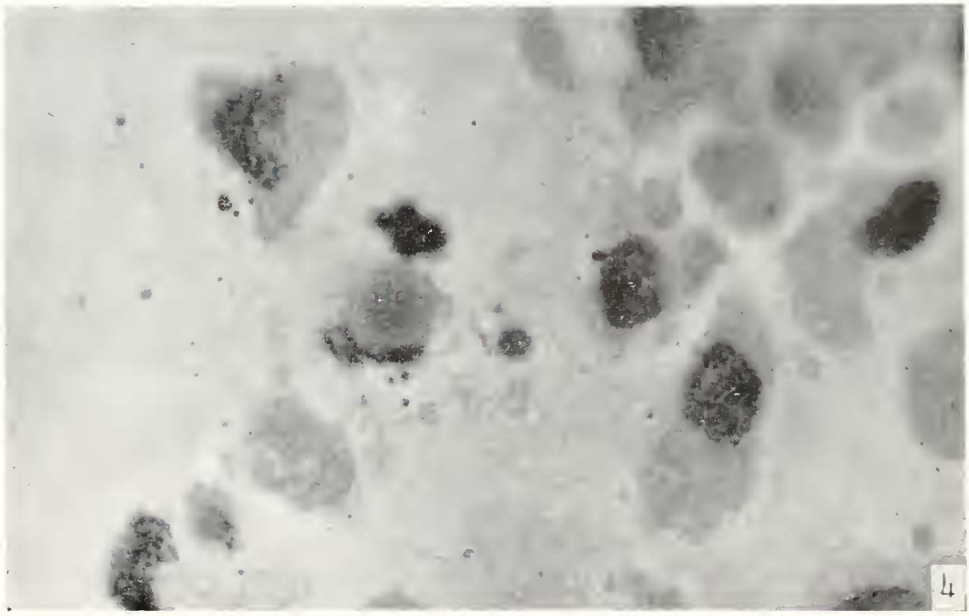


FIGURE 3. Smear preparation of ascitic fluid from the same mouse 72 hours after intraperitoneal injection of spleen extract. Small solid tumors are beginning to form around islands made up of aggregations of tumor cells and leucocytes. Ca. 400 ×.

FIGURE 4. Smear preparation of ascitic fluid from a mouse 48 hours after intraperitoneal injection of spleen extract (150 mg./ml.) showing total degeneration of almost all tumor cells. (In such cases only about 5% of the tumor cells remained viable.) Ca. 1100 ×.

containing approximately 150 mg. of solids ml., the percentage of apparently viable tumor cells remained very low at 48 hours (only 5.5% had a normal appearance; see Fig. 4), since most of the cells were shrunken and coagulated.

Morphological changes. The degenerating tumor cells just mentioned showed cytological changes consisting of nuclear shrinkage and pycnosis and vacuolization of the cytoplasm. These changes are essentially the same as those observed in solid tumors on the third day after beginning of spleen injection (Diller and Watson, 1949) and those reported by MacFarlane (1948) after storage of tumor cells in concentrated spleen extract. No evidence of interference with the spindle mechanism was observed in our experiments with ascites tumors, though Katzberg (1952) reported this phenomenon as occurring in the first 24 hours of storage in his splenic preparations, and the percentage of multipolarity was increased in solid S-37 treated by us with low concentrations (Diller and Watson, 1949).

Survival. Maximum survival time was attained by injecting 0.25 cc. of the Lilly extract four times daily at 6-hour intervals, beginning either immediately after inoculation of the host with ascites tumor, or on the third day post-inoculation. This induced regression of the ascites swelling in 16 out of 60 animals treated, though solid masses persisted in the abdominal cavities of these mice. Large single masses were not noticeably affected by continued spleen extract injection, but grew like a trocar-introduced solid tumor, resulting in death of 10 of the 16 solid-tumor-bearing animals in from 18–24 days (mean survival 20.7 days). Control animals bearing untreated ascites tumors died 7–9 days (mean 7.1 days) after inoculation. In the group of 16 mice in which solid tumors developed, complete resorption of smaller masses was induced in 6 animals under continued spleen therapy. The host animals were still tumor-free six months later, when they were sacrificed. It will thus be seen that even the most effective samples of extract with respect to mitotic inhibition and tumor cell destruction did not improve the chance of survival of the host unless the ascitic fluid was absorbed and the neoplasm converted to the solid form. Of these solid tumors, about one-third regressed under continued spleen extract injection. Since the fate of small solid sarcoma 37 in mice treated with similar spleen extracts is already known (Diller and Watson, 1949) further studies on larger numbers of animals were not carried out.

In most of our experiments ascites swelling was already visible when treatment was begun. This procedure was followed in order to ensure actual presence of neoplasia. However, studies were made also on the effect of injecting the extract prior to inoculation with ascites fluid. If no further spleen extract injections were made after inoculation of the tumor, the course of development was unaffected. On the other hand, when injection of the extract was resumed on the day of inoculation of the tumor, all the animals died with hemorrhage in the peritoneal cavity before ascites swelling appeared. Non-tumor-bearing control mice suffered no casualties when subjected at the same time intervals to interrupted dosage of the same magnitude. Simultaneous injection of extract into the peritoneal cavity of mice at the time of tumor inoculation did not prevent the development of the ascites tumor.

Discussion

There are two types of reaction to injection of the splenic extract into mice bearing sarcoma 37 ascites:

1. *An inflammatory one* involving a sharp increase in the number of polymorphonuclear leucocytes (Fig. 2) and a less marked, but more persistent, increase in the number of lymphocytes present in the ascitic fluid. The same phenomena were observed in the case of mice bearing solid sarcoma 37 (Diller and Watson, 1949), since on the third day after beginning of treatment there was considerable infiltration of the tumor tissue by leucocytes, including an increased number of macrophages.

2. *Inhibition of mitosis* in all of the tumors and varying amounts of necrotic change (depending on the sample of spleen extract employed) with consequent reduction in tumor cell population accompanied by formation of small solid tumors.

In addition to reports of spleen-extract-induced regression of already developed tumors, noted at the beginning of this article, there have been numerous accounts of the inhibitory effect of spleen extract on establishment of tumor transplants or on experimental tumor induction. For example, Sugiura (1938) found that aqueous extracts of several organs of the rat had a depressant action on growth of mouse sarcoma 180 and on Flexner-Jobling rat carcinoma when, prior to implantation, the tumor fragments were stored in the extracts at 4–5° C. before implantation into mice. He reported also cessation of mitosis *in vitro* with degenerative nuclear changes under the same experimental conditions.

Yun (1950a, 1950b, 1950c) reported that pre-injection of mice with splenic extract inhibited development of tumors in mice painted with methylcholanthrene and also that extirpation of the spleen from A strain mice furthered the development of methylcholanthrene tumors.

When Nuttini (1951) pre-injected rabbits with rabbit spleen pulp or cell-free filtrate thereof, 66% of the host animals were protected against development of Brown-Pearce carcinoma. Therapeutic administration of the filtrate also induced total regression in a significant percentage of the animals treated.

In 1952 Foley reported retardation and/or regression of lymphosarcoma in C3H mice implanted subcutaneously with cell suspensions of spleen from an alien mouse strain. Also according to Lorenz *et al.* (1953), spleen shielding and bone marrow injections effectively protect mice against x-ray induction of lymphoid tumors. When C57 Black mice were given four doses of 225 r without spleen protection 70% of the mice developed lymphoid tumors but after the same amount of irradiation in a spleen-shielded group tumors appeared in only 3% of the animals. On the basis of studies of tumor tissue and spleen tissue grown *in vitro* Pollard and Bussell (1953) showed that spleen tissue from the host asserted no destructive effect on its own tumor tissue though similar tissue from the spleen of a rat that had sloughed a tumor had a rapid destructive effect.

That the inhibition of mitotic processes is not confined to neoplastic tissue in contact with splenic extract was indicated by Fardon *et al.* (1948) who reported partial inhibition of mitosis *in vivo* in the crypts of Lieberkühn of mice injected with beef spleen extract; and Hoffman (1940) found that extract of kidney, spleen, liver and lung definitely inhibited growth of fibroblast colonies *in vitro*.

Rohdenburg and Nagy (1937) showed that *both* inhibitory and stimulatory materials are present in rabbit and human spleen. The inhibitory agent was found in the initial acetone-soluble fraction. In our earlier experiments (1949) spleen, liver, thymus, lymph nodes and, occasionally, adrenal tissues of the treated mice were stimulated to mitotic activity by doses that were capable of causing regression

of solid tumors. This led us at that time to postulate that the suppressive effect on tumors was probably due, not to direct cytolysis, but to stimulation of the hemopoietic organs and the adrenal cortex. Furthermore, extract of low concentration appeared to stimulate mitosis in solid tumors, resulting in polyploid cells (as judged by numbers of nucleoli) and multipolar spindles. It is possible that these were, in fact, damaged cells which would eventually have become inviable. Bearing on this point are the findings with respect to ascites tumors injected with the 50 mg./ml. concentration (Table II) where inhibition of mitosis was obtained more rapidly with the low than with the high concentration. However, the 150 mg./ml. preparation produced drastic cell damage and eventual destruction of a much higher percentage of tumor cells (only 5.5% viable after 48 hours) though the host animals usually did not survive more than an additional day and died ahead of the controls. Non-tumor-bearing control mice tolerated injections of the same magnitude without adverse reaction, which suggests that toxic substances were being released by the degenerating cells. Klein (1951) reported that if cells are damaged before or after introduction into the peritoneal cavity, the course of events will be the same as though low cell numbers were originally injected, *i.e.*, there occurs a very intense inflammatory reaction after which tumor cells disappear completely from the peritoneal fluid, or are present in relatively low numbers and the animals die much later with solid tumors. Apparently this is what occurred in our own experiments in 10% of the animals.

Cook and collaborators (1951) attempted to determine the mechanism by which tissue extracts operate in growth processes and reported that spleen extract has an inhibiting effect on certain enzyme systems known to function in cellular respiration. Aqueous homologous cell-free spleen preparations were the most effective in stimulating oxygen uptake. Water-soluble spleen extract caused depression in various concentrations, except those from 25–10 mg./ml., which produced stimulation. In general, neoplastic tissues showed greater changes than did normal tissues.

Whether or not splenic extract exerts a direct cytolytic action on tumor cells is still not proven since the evidence from ascites tumors is that the host participates through leucocytic response. However, the experiments herein described or reviewed appear to indicate that there are substances present in splenic extract capable of suppressing mitosis for varying periods of time and of inducing cytolysis in tumors, while at the same time stimulating the hemopoietic system of the host.

Summary

1. The injection of a single dose of aqueous beef spleen extract containing 50 mg.–150 mg./ml. of solid materials directly into the peritoneal cavity of mice bearing ascites sarcoma 37 resulted in inhibition of mitosis for a period of at least 48 hours, and in some cases in complete cessation of mitosis that persisted for even longer periods. Mitotic inhibition was accompanied by a marked inflammatory reaction. Cell counts per cc. of ascites fluid were lowered and the percentage of viable tumor cells decreased, partly as the result of cessation of mitosis and necrotic change, and partly through dilution with white blood cells.

2. The reduction of tumor cell population was accompanied by the aggregation of tumor cells into islands which presumably served as centers for the formation of

solid tumors. When the tumor masses thus formed were large, no regression was observed, though survival time was considerably lengthened; however, small masses were completely resorbed under continued spleen extract therapy in a third of the animals whose tumors had become solid under spleen extract injection (10% of the total number of animals treated). No reduction in ascites swelling unaccompanied by the formation of solid tumors was observed.

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